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Editorial

AGRICULTURAL IMPROVEMENT COUNCIL

ELSEWHERE in this issue of THE VETERINARY JOURNAL we publish an announcement by the Minister of Agriculture that he has set up a new body—the Agricultural Improvement Council. On April 3 in the House of Commons he stated: "... We are impressed with the need of taking more active steps to see that the results of research are made known to the ordinary farmer and given greater opportunities of establishing themselves in everyday practice. To this end I propose to appoint a body for England and Wales which will be responsible to me directly and which I hope will command as much respect in the agricultural world as the Agricultural Research Council does in the scientific world. The functions of the new body will be twofold. On the one hand, it will be concerned to devise methods for seeing that all promising results of research are applied as rapidly as possible to the problems of agriculture and are incorporated in the practice of the everyday farmer. On the other hand, it will keep me informed—so that I can advise the Agricultural Research Council—on any problems of agricultural practice which it considers should be the subject of scientific investigation. . . . As Scottish problems do in many respects differ considerably from those of England and Wales, my right honorable Friend the Secretary of State for Scotland will appoint a similar body for Scotland.

I should add that it is hoped that members of the Agricultural Research Council will be appointed both to the English and to the Scottish bodies so as to preserve as close a liaison as possible."

The Minister is to be congratulated on forming this new body for, as we have remarked on more than one occasion in this Journal, the end of all research is its practical application. We as a profession are concerned mainly with the health and disease of livestock. It will be noted that members of the profession are included in the membership of the new Council. Too often, it is feared, have the results of research work been greatly delayed in their application on the farms. It cannot be too strongly emphasized, of course, that undue haste on somewhat flimsy evidence in the adoption of methods for the treatment and control of disease may lead to disastrous and disappointing results. It will, we take it, be a function of the Agricultural Improvement Council together with the Agricultural Research Council to satisfy itself that the methods are of practical value and then to devise means whereby their application is brought about in a minimum of time. It is also gratifying to note that close liaison is to be observed with the Agricultural Research Council. With these two bodies working in collaboration it is safe to forecast that, among other aspects of agriculture, the health and disease of livestock will receive the attention due to them and the results of research work concerning them will be carried on to the farms with the least possible delay.

The veterinary profession will have an important part to play in all this work. It is more important to-day than at any time in the past that the livestock throughout the country should be maintained in a healthy condition. The veterinary practitioner must play a large part in ensuring that disease is controlled to the greatest possible extent. In order to do this he must keep himself well acquainted with the latest methods of control and he must be given the necessary facilities to practise his knowledge. The veterinary profession must devise means of disseminating new knowledge among its members. The veterinary practitioner must be given the opportunity of dealing with disease in flocks and herds before their incidence is such that it may be uneconomic to practise any method of control, however good it may be. We are confident that any assistance which the profession can render to the Agricultural Improvement Council will be freely given and that any recommendation made by the Council affecting the profession will be willingly undertaken.

General Articles

THE STANDARDIZATION OF JOHNIN

2. Purification of Johnin and its Comparison with an Unpurified Product.

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In a previous paper (Glover, 1941) it was shown that guinea-pigs injected intraperitoneally with suitable doses of *M. paratuberculosis* in liquid paraffin were rendered sufficiently skin sensitive to permit a comparison of various johnins, all of which had been prepared in synthetic medium and concentrated by heat. In the absence of a recognised standard preparation, a particular johnin (J.15), selected at random, was used at each test and the potency of other johnins determined in terms of this batch. It was recognised that the conclusions which were drawn were purely tentative and might require revision if subsequent study showed that this johnin, by reason of too great a potency or of insufficient strength, proved to be unsatisfactory. The problem of the selection of a johnin of suitable activity for use in cattle practice is complex, since, in addition to the necessity for preparations of adequate potency, questions of specificity have also to be taken into account. The final decision as to the type of johnin which can be accepted as a standard must depend on extensive trials in known positive and negative cattle, but such experiments are costly and time consuming. The absence of a small animal which can be used for preliminary trials on different johnins has undoubtedly checked progress, and it is hoped that the guinea-pig test which has been described will permit more extensive preliminary trials on johnins and thus reduce the number of cattle tests which would otherwise be required.

In the assay of tuberculin the acceptance of a standard preparation of "O.T." has led to a high degree of uniformity in the methods of conducting and interpreting the test in the guinea-pig. Still further improvements have been effected by the introduction of precipitated tuberculin protein and in particular of purified protein derivative (P.P.D.), (Seibert 1934). Many careful experiments in guinea-pigs have permitted the evaluation of O.T. in terms of P.P.D., which, in many respects, presents advantages over a liquid preparation. Doig and collaborators (1938) have found that, in general, when redissolved at a concentration of about 5 mg. per ml., successive batches of P.P.D. have been equal in potency to the International Standard old tuberculin on guinea-pig intracutaneous tests. They point out that it is of almost constant potency on

a weight basis, is very stable in a dry state and is of a non-sensitizing nature. In their view it is particularly suitable for use in large-scale surveys where uniformity in the amount of active principle injected is essential.

The main object of the present investigation was to determine whether it was possible to produce a johnin P.P.D. and then to ascertain the value of serial batches in terms of J.15, using the intracutaneous test in sensitized guinea-pigs. It was thought that if any degree of uniformity was obtained, it might be considered sufficiently encouraging to warrant further tests in Johne affected cattle.

Preparation of P.P.D. Johnin.—It was decided that in addition to the process devised by Seibert (1934), other methods of obtaining the protein derivatives should be tried. Dry powders were therefore secured by an adaption of Gough's method (1934) for the preparation of tuberculin protein, and by precipitation with ammonium sulphate.

P.P.D. Johnin (*Seibert's method slightly modified*).—The " teps " strain of *M. paratuberculosis* was grown on Henley's synthetic medium contained in Erlenmeyer flasks (250 ml. per flask) for about three weeks. The cultures were then steamed for one hour and the organisms removed by passing the liquid through paper. The clear fluid was concentrated to 1/5th its volume by heat after the addition of glycerol in the proportion of 1.2 ml. per 100 ml. and was filtered while hot through a small Berkefeld candle. As the addition of one per cent. phenol water was found to produce a fairly heavy precipitate which contained a certain proportion of active principle other diluents were tried. It was ascertained that distilled water containing 1-25,000 merthiolate gave satisfactory results, since it inhibited bacterial growth but did not cause any significant precipitation. The dialysable constituents were then removed by filtration under pressure through a collodion ultrafilter. The membrane was prepared from necol collodion evaporated to dryness and redissolved in glacial acetic acid to make a 15 per cent. solution. In the early attempts at producing johnin P.P.D. more dilute collodions were tried (7 per cent. to 12 per cent.) but these strengths were unsatisfactory since a small proportion of the active principle passed through the candle and was detected in the filtrate by the addition of trichloracetic acid. The collodion film was supported on acid-resistant Dalton candles. Washing was continued with merthiolate water until the filtrate was almost free from chloride, after which the solution was reduced to a small bulk. The addition of sufficient 50 per cent. trichloracetic acid solution to give a final concentration of 10 per cent. produced a heavy grey precipitate which was collected by centrifugation and purified by redissolving in 1 : 1,000 $NaHCO_3$ solution and reprecipitating with trichloracetic acid. The final stages of washing with acid and drying with ether were in accordance with the instructions of Seibert.

Serial batches of johnin P.P.D. prepared in this matter were light brown

or pale yellow powders which were readily soluble in dilute alkaline solutions.

Gough's Method.—A very satisfactory method of obtaining tuberculin protein devised by Gough (1934) was based upon the adsorption of the active principle to precipitated benzoic acid. A slight modification of this process was applied to the preparation of johnin protein. After concentration by heat and preliminary purification on a collodion membrane, a solution of sodium benzoate in caustic soda (1 g. Na benzoate in 5 ml. of 2N. NaOH) to every 100 ml. of liquid was then added and 3N. HCL rapidly poured in with constant stirring. About 10 ml. of acid per 100 ml. was required to produce complete precipitation of the benzoic acid. An abundant heavy precipitate was formed which was collected by centrifugation, thickly smeared over the inside of a wide centrifuge tube and dried "in vacuo" as rapidly as possible. The benzoic acid was dissolved by liberal treatment with successive quantities of acetone, the sediment redissolved in 1-1,000 $NaHCO_3$ and reprecipitated on benzoic acid. After further treatment with acetone the sediment was washed and dried with ether. A very friable, yellowish-white powder was obtained.

Ammonium Sulphate Precipitation.—Dried powders were also obtained by methods which have been described in a previous paper on the preparation of synthetic medium tuberculins and johnins (Glover, 1932). The active principle was separated from heated or unheated synthetic medium johnin by the addition of ammonium sulphate to saturation point. The precipitate was collected, redissolved in one per cent. sodium bicarbonate solution and dialysed until free from salts. The johnin protein was then absorbed on benzoic acid from which it was liberated by treatment of the sediment with acetone followed by ether. Previous work on tuberculin protein (unpublished) has shown that the weight of active substance obtained by the ammonium sulphate method is closely governed by the reaction of the liquid. The highest yields have occurred within the limits of pH 5.6—pH 6.4, smaller amounts being obtained outside this range in either the acid or alkaline direction. Weight for weight, no appreciable difference in potency was detected as between the different samples. The conclusion was therefore reached that when the reaction was adjusted to the range which induced maximum precipitation no extraneous substance came out of solution. Similar observations have been made in regard to johnin protein.

A typical example of the effect of different pH values on the yield resulting from ammonium sulphate precipitation is revealed by the following experiment. Four litres of synthetic medium culture was passed through paper and the filtrate concentrated to one-fifth its volume, 50 ml. of glycerin having been added prior to the heat treatment. After filtration through a Berkefeld candle the fluid was divided into four equal parts of 200 ml. each, which were adjusted by the addition of appropriate amounts of acid or alkali to give solutions with reactions of pH 7.0, 6.4, 5.6, and 4.8 respectively.

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Ammonium sulphate was then added to saturation point, the four precipitates collected, dissolved in one per cent. sodium bicarbonate, dialysed and recovered by the benzoic acid method. Table I gives the yields of dry powder obtained from each 100 ml. of dilute johnin, an allowance having been made for fluid lost through evaporation during the period of growth. The superiority of a reaction of pH 5.6 on the amount of dry material recovered is clearly indicated. The yield obtained from another portion of the same johnin by concentration on an ultra-filter and separation of the active principle by the addition of some benzoic acid is also shown in the same Table. In this instance the yield per 100 ml. of original fluid was less than that obtained by a preliminary precipitation with ammonium sulphate at pH 5.6.

TABLE I

Effect of pH Reaction on the Yield of Johnin PPD by the Ammonium Sulphate Method

No. of johnin.	*Method of preparation.*	*Total weight.*	*Weight per 100 ml. dilute johnin.*
		mg.	*mg.*
JP.6	Benzoic acid	160.0	16.0
JP.7	Ammonium sulphate pH 7.0	90.0	9.0
JP.8	„ „ pH 6.4	105.0	10.5
JP.9	„ „ pH 5.6	185.0	18.5
JP.10	„ „ pH 4.8	85.5	8.55

Table II shows the weights of a number of other preparations obtained by ultrafiltration followed by trichloracetic or benzoic acid precipitation. Attention may be drawn to a variation in the proportion of dry substance separated from 100 ml. of fluid on successive occasions. This may have been attributable in part to loss of material during the process of washing on the collodion membrane, and in part to fluctuations in the amounts of active principle elaborated by the organism during growth.

It will be noted that the percentage amounts of dry protein derivative obtained from successive batches of johnin were appreciably less than those recorded by other workers in respect of tuberculin protein. Seibert (1934) gives a table showing that the yields of PPD from six samples of tuberculin ranged from 0.075 g. to 0.053 g. per 100 ml., while a seventh sample amounted to 0.021 g.

When dilutions of dry P.P.D. were required for immediate use the powders were brought into solution by taking weighed amounts (usually 10 mg.), grinding in a dry mortar to a fine powder and running in one per cent. sodium bicarbonate solution drop by drop until 20 ml. had been added. The solution, which at this stage was very slightly opalescent, was gently warmed to 50 deg. C. until it became limpid and was adjusted to pH 7.4 by

the addition of 5 per cent. HCl in the presence of phenol red. The volume was then brought to 40 ml. with neutral distilled water. The desired dilutions were prepared from this mother solution.

TABLE II

(1) *Amounts of Johnin PPD obtained by Trichloracetic and Benzoic Acid methods.*

(2) *Potency in terms of* J.15.

No. of johnin.	*Method of preparation.*	*Total weight.* mg.	*Weight per* 100 *ml. dilute johnin.* mg.	*Potency in terms of* J.15. mg.
JP.1	T.C.A.*	500	13.5	0.004
JP.2	T.C.A.	360	14.6	0.004 — 0.006
JP.3	T.C.A.	410	10.25	0.004
JP.5	T.C.A.	200	20.0	0.003 — 0.004
JP.11	B†	895	21.3	0.003
JP.12	T.C.A.	610	15.2	0.003 — 0.004
JP.16	B	765	22,07	0.004 — 0.005
JP.18‡	B	550	1.64	0.008
JP.22	T.C.A.	570	20.9	0.003
JP.24	T.C.A.	740	19.8	0.004
JP.26	T.C.A.	650	16.5	0.003

T.C.A.* = trichloracetic acid precipitation.
B† = benzoic acid precipitation.
JP.18‡ from unheated johnin.

Tests of Johnin P.P.D. in the Normal Guinea-pig.—Before describing the experiments on allergic guinea-pigs, it may be stated that a number of normal animals has been tested with johnin P.P.D. prepared by the three methods outlined above. It has already been shown (Glover, 1941) that a dose of 0.2 ml. of heat-concentrated synthetic medium johnin injected intracutaneously into the non-sensitised guinea-pig may give rise to a slightly hæmorrhagic plaque measuring from 3 mm. to 5 mm. in diameter, whereas a 1 : 5 dilution is without effect. The protein derivatives in concentrations of 10 mg., 5 mg., 3 mg. and 1 mg., per ml. were injected intradermally on one side of six normal guinea-pigs, and a heat concentrated, synthetic medium johnin, undiluted and in dilutions of 1 : 5, 1 : 10 and 1 : 20 on the other. Two batches, both of which had been prepared by ultrafiltration followed by precipitation with trichloracetic acid, gave a transient roseate reaction at a concentration of 10 mg. per ml., but were without effect when diluted to 5 mg. per ml.; otherwise these purified products failed to produce a detectable reaction on the normal guinea-pig skin.

Comparison of Heat-concentrated Synthetic Medium Johnin with P.P.D. Johnin.—The method of sensitising guinea-pigs for the intracutaneous johnin test has been described in detail (Glover, 1941): briefly, the surface growth from a culture on liquid synthetic medium was added to sterile liquid paraffin to give a suspension of 60 mg. per ml. and was injected intraperitoneally into guinea-pigs weighing about 500 g., each animal receiving 0.5 ml. After an interval of seven to eight weeks an orientating test was performed on three guinea-pigs. The heat-concentrated synthetic medium johnin (J.15), which has been arbitrarily selected as a standard, was made up in dilutions of 1 : 1,000, 1 : 2,000, 1 : 3,000, 1 : 4,000, 1 : 5,000, 1 : 6,000, 1 : 7,000 and 1 : 8,000, which were injected intracutaneously into each guinea-pig in doses of 0.2 ml. The reactions were read at the 48th hour. These preliminary trials showed whether the animals were sufficiently sensitive for the main test. In practice, successive groups of guinea-pigs reacted in a fairly uniform manner, dilutions of 1 : 1,000 and 1 : 2,000 giving large swellings measuring from 20 to 26 mm. with hæmorrhage and necrosis, the intensity of the reactions diminishing progressively with the amount of active principle. Nevertheless, in some instances, the responses to the 1 : 7,000 and 1 : 8,000 dilutions were almost inappreciable or were not well defined in every animal. On account of these slight fluctuations in individual sensitivity it was difficult to select a single dilution which would reveal the greatest variation resulting from slight differences in potency of the preparations under test. In order, therefore, to minimise the effect of uneven sensitivity each batch of guinea-pigs used in the main test was divided into three groups assigned to dilutions of 1 : 2,000, 1 : 5,000 and 1 : 8,000 respectively.

The first experiments were concerned with the comparison of heat-concentrated synthetic medium johnin with several samples of johnin P.P.D. In performing these tests appropriate dilutions of the heat-concentrated johnin, J.15, were prepared, together with the same dilutions of a 50 per cent. and a 25 per cent. solution. These were compared with similar dilutions of P.P.D. in amounts varying from 10 mg. to 1 mg. per ml. The preliminary trials indicated that in a strength of 10 mg. per ml., P.P.D. johnin was at least twice as potent as J.15, while a solution of 1 mg. per ml. was from 50 per cent. to 20 per cent. below the value of the same johnin. The reactions were judged (1) by a measurement of the swelling from which the average diameter was calculated, and (2) by a series of positive signs representing the intensity of the reaction, with special reference to hæmorrhage and necrosis. The figures obtained in all the guinea-pigs under these two headings were added together and the totals were compared with one another. In Table III the detailed figures are given, but in subsequent tables, only the sums of the readings are recorded.

Table III gives the result of a test on 14 guinea-pigs using J.15 (undiluted,

75 per cent., 50 per cent., and 25 per cent.) on the one hand, and JP.11, a P.P.D. prepared by the benzoic acid method in strengths of 5, 4, 3, and 2 mg. per ml. respectively. It will be seen that a solution of JP.11 containing 3 mg. per ml. evoked a response equal in intensity to that of J.15 undiluted; 4 mg. per ml. was slightly more active, while 2 mg. per ml. was less potent. Three tests have been completed with this preparation, one of which gave a value of 3 mg. per ml., while the others fell between 3 mg. and 4 mg. per ml. as the equivalents of J.15.

TABLE III

Comparison of Standard Heat Concentrated Johnin (J.15) *with Johnin P.P.D.* (JP.11)

Johnin.	G.-pig	1	2	3	4	5	6	7	8	9	10	11	12	13	14	*Total.*
		1 : 8,000				1 : 5,000					1 : 2,000					
J.15 undil.	D	10	10	5	8	12	14	8	10	12	20	22	18	18	20	187
	R	2	2	1	1	2	3	1	2	3	4	5	4	4	6	40
J.15 75%	D	8	5	10	12	12	12	14	12	10	20	22	18	18	18	191
	R	1	1	2	2	3	3	2	2	3	5	6	4	3	4	41
J.15 50%	D	5	5	0	8	5	8	8	5	5	18	16	18	14	16	131
	R	1	1	0	1	1	2	1	1	1	3	3	4	3	3	25
J.15 25%	D	0	0	5	0	5	0	5	5	0	10	8	10	5	8	61
	R	0	0	1	0	1	0	1	1	0	2	2	2	1	2	13
JP.11 5 mg.	D	14	10	8	10	14	16	12	10	10	24	26	20	18	18	210
	R	3	1	1	2	3	3	2	2	2	7	6	6	4	4	46
JP.11 4 mg.	D	12	12	8	5	14	14	10	12	14	22	24	22	20	16	205
	R	2	2	1	1	2	3	2	2	3	5	5	4	4	4	40
JP.11 3 mg.	D	5	10	8	7	12	16	10	8	10	20	20	18	20	16	180
	R	1	2	1	1	2	3	2	1	2	5	4	4	4	3	35
JP.11 2 mg.	D	5	5	5	6	10	14	12	10	10	20	18	18	16	18	167
	R	1	1	1	1	1	2	2	1	1	4	3	3	2	3	26

D = mean diameter of reaction in mm.
R = degree of reaction (figures 1 to 7 represent increasing extent of involvement of skin. Reactions of 6 and 7 show intense hæmorrhagic centre or necrosis).

A preliminary test of J.15 and JP.22, prepared by the trichloracetic acid method, showed that in a strength of 5 mg. per ml. JP.22 possessed a potency of approximately 1½ times the value of J.15. A second test was performed in which solutions containing 5 mg., 4 mg., 3 mg., and 2 mg. per ml. respectively were compared with J.15, J.15 (50 per cent.) and J.15 (25 per cent.). The results (Table IV) show that in strengths of 5 mg., and 4 mg., JP.22 was more potent than J.15 undiluted, while 3 mg. was about equal in value to it.

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The solution containing 2 mg. was slightly less potent than J.15 undiluted but gave reactions equal in intensity to a 50 per cent. dilution.

TABLE IV

Comparison of J.15 *and* JP.22

Johnin.	*Total reaction on* 13 *guinea-pigs.*	
	D	R
J.15	212	54
J.15 (50%)	163	40
J.15 (25%)	87	22
JP.22 5 mg.	223	58
JP.22 4 mg.	222	57
JP.22 3 mg.	207	50
JP.22 2 mg.	174	42

D = mean diameter of reactions in mm.
R = degree of reaction.

Very similar results were obtained when a second heat-concentrated johnin (J.19) was employed. Preliminary trials showed that J.19 was not quite so potent as J.15 but it could not be distinguished from a 50 per cent. dilution of the latter, and was definitely stronger than a 25 per cent. dilution. Comparative tests of J.19 and JP.22 show that a solution of JP.22 containing 2 mg. per ml. was equivalent to J.19; in view of the slight superiority of J.15 over J.19 these results might have been anticipated.

Every fresh batch of P.P.D. johnin which has been prepared has been compared in serial dilutions with J.15. The results are set out in Table II and show the value of successive samples of these P.P.D.s in terms of J.15. It is obvious that the various methods of preparing johnin P.P.D. have given products which have shown little difference in their potency.

Attention may be directed, however, to JP.18, which was not very active. It is almost certain that the low potency of JP.18 was dependent upon the fact that the johnin was not subjected to any form of heat treatment.

After removal from the incubator the liquid portion was syphoned from the flasks, passed through a Berkefeld V candle, and concentrated to a quarter of its volume by filtration under negative pressure through a collodion membrane of the usual type. Distilled water was then added to the fluid until the dialysate was free from salts: in the course of this process the liquid was reduced to about one-twentieth of its original volume. The johnin protein was separated by the benzoic acid method and obtained as a dry powder. The yield of active substance, which may be regarded as the johnin counterpart of Seibert's unheated tuberculin protein (T.P.T.) was poor. (See Table II.) Tests on the dialysate indicated that in the early stages of filtration an appreciable amount of protein, precipitable by Esbach's reagent, was passing through the collodion membrane, but as filtration proceeded the proportion escaping steadily diminished and finally ceased.

Doubtless the restricted yield of dry powder from this particular batch was partly attributable to the loss of active principle through the candle. In addition, however, to a poor yield of johnin protein the final product was of a lower potency than any of the P.P.D.s prepared from heat-concentrated johnin since, in order to obtain the equivalent of J.15, it was necessary to increase the strength of the solution to 8 mg. per ml. It would appear, therefore, that quite apart from the danger of sensitization which might arise if undenatured proteins of this type were used for serial tests, the preparation of P.P.D. from unheated johnin is not an economical proposition.

Keeping Qualities of Johnin P.P.D.—Like tuberculin P.P.D., johnin proteins in the dry form have proved quite stable. One of the early preparations (JP.3) was dried to constant weight over P_2O_5, distributed into ampoules which were evacuated and sealed. This batch has been tested from time to time and has shown no appreciable loss after two years, three months. Solutions of johnin P.P.D. also retained their potency almost unimpaired for some time at cold-room temperature but tended to deteriorate at 37 deg. C. The loss in activity was estimated by preparing a solution of JP.3 in a buffered solution. 50 mg. was dissolved in 25 ml. of one per cent. $NaHCO_3$ and was adjusted to pH 7.6 by the addition of 25 c.c of 50 per cent. glycerol containing 1 : 1,000 citric acid and 1 : 10,000 merthiolate. The solutions were distributed in small ampoules which were sealed and stored at 37 deg. C., room temperature (14 deg. C. to 22 deg. C.), and in the cold store. Loss in potency was determined by the injection of suitable dilutions in sensitized guinea-pigs at intervals of 60 and 175 days respectively, with the results shown in Table V.

TABLE V

Loss in Potency of JP.3 *in Solution on storing at* (1) 37 *deg. C.;* (2) *Room Temperature;* (3) *Cold Room Temperature*

J.15		133	176	204
J.15 50%		113	144	176
J.15 20%		99	98	126
	Temp.:	0 days	60 days	175 days
JP.3 5 mg. per ml.	37 deg. C.	148	86	16
JP.3 5 mg. per ml.	R.T.	148	177	128
JP.3 5 mg. per ml.	C.R.	151	198	210

R.T. = room temperature.
C.R. = cold room.

Figures represent sum of diameter of reaction in 10 to 13 guinea-pigs.

The initial test showed that the P.P.D. solution was more potent than J.15. Previous trials had given the value of 3 mg. per ml. of JP.3 as equal to J.15. At the sixtieth day period there was no significant loss in potency at 0 deg. C., but at room temperature the solution had a value about equal to J.15, while at 37 deg. C. there had been a loss of activity of at least 75 per cent. After 175 days the cold room preparation was still slightly superior to standard, but the solution kept at room temperature had now fallen to less than 25 per cent., while at 37 deg. C. the amount of active principle was so small that it was only just detectable in the strongest solution which was used.

These results appear to show a more rapid deterioration in potency than has been reported by Douglas and Hartley (1934) for Old Tuberculin. A possible explanation of the divergent result may be that a different type of buffer solution was used by these workers, who diluted their O.T. with a borax-boracic acid buffer solution, whereas a bicarbonate-citrate mixture was used for the solution of the P.P.D. Other experiments are now being undertaken with johnin P.P.D. in borax buffer.

The resistance of johnin P.P.D. to high temperatures was also examined. Preparation JP.3 was again selected, tests being made both on the dry powder and on a solution. One part of the powder was sealed in ampoules, the contents of which were dissolved after heating to give a solution containing 5 mg. per ml.; the second part was made up in a solution of 5 mg. per ml. in a bicarbonate glycerol citrate solution, as in the previous experiments, except that the merthiolate was omitted, and was then distributed into ampoules each containing 5 ml. The samples were heated at 60 deg. C., 80 deg. C., or 100 deg. C., for two hours and for ten hours respectively by total immersion in a water bath. Loss in potency was determined by the intracutaneous test on sensitised guinea-pigs, a comparison being made with a fresh solution of the same strength.

It was noted that neither the dried powder nor the solution showed a detectable loss in value after heating for two hours. In the case of the longer period of ten hours, heating to 80 deg. C. and to 100 deg. C. effected a drop in potency, both of the powder and the solution, which, in the case of the former, was not more than 50 per cent., but was approximately 75 per cent. of the latter. This result was rather surprising, but the probable explanation lies in the observation that after prolonged heating at 80 deg. C., or over, P.P.D. johnin in powder form loses a part of its solubility: even when prepared in a strength of one mg. per ml. a completely limpid fluid was not obtainable under these conditions.

Tests in Cattle.—A number of P.P.D. johnins have been tested in a small herd of cattle, known to be free from Johne's disease, by the double intradermal method (dose 0.1 ml.). On the first occasion nine cows and heifers were injected with a solution of JP.11 containing 6 mg. per ml. In six

animals the reactions were clear-cut negatives, the swellings being circumscribed with a final mean increase for the group not exceeding 3 mm. In the remaining three, however, there were increases of 3.5 mm., 3.8 mm., and 4 mm., respectively, and in each case the reaction, which was slightly doughy, could only be described as indefinite. Five of the animals were subsequently tested with solutions of JP.11 containing 5 mg., 4 mg., 3 mg., 2 mg., 1 m.g., and 0.5 mg. per ml. respectively. As a control the heat-concentrated johnin JP.15 was also injected undiluted, and diluted with an equal quantity of saline (Table VI). Because of differences in sensitivity in varying areas of the skin, the sites of injection were changed in each animal.

TABLE VI

Tests of Negative Cattle with Johnin P.P.D. JP.11 *and with* J.15

No. of	JP.11 *mg. per ml.*						J.15	
animal.	5	4	3	2	1	0.5	*undil.*	50%
M.H. 129	3.0*	3.0	2.0	2.0	1.5	1.5	3.0	2.0
M.H. 133	3.5	3.0	2.0	3.0	2.0	2.0	3.5	2.0
M.H. 165	2.5	2.0	2.0	1.5	1.0	1.0	3.0	2.5
M.H. 166	3.0	2.5	3.0	2.0	2.0	1.5	3.0	2.0
M.H. 177	3.5	3.0	2.0	2.0	1.5	2.0	4.0	2.0

* Figures represent mean increase in mm. at 72nd hour.

In general, doses of 5 mg. and 4 mg. gave swellings of the size of a large pea; they were not quite so pronounced as those resulting from J.15. There was little difference between 3 mg., 2 mg., 1 mg., and 0.5 mg., the reactions varying from a small pea to a slight thickening of the skin which was just appreciable. The same nine animals were later subjected to a test in which JP.22 was injected on one side of the neck in strengths of 4 mg., 3 mg., 2 mg., and 1 mg. per ml., while similar amounts of JP.24 were inoculated on the other side. Very little difference could be detected in the character of the reactions induced by the four dilutions of the two products.

So far it has not been possible to undertake any extensive tests in positive cattle. By courtesy of Mr. G. W. Dunkin, however, a small test was undertaken on seven cattle, four of which were known to react to johnin, one was doubtful and two were negative. The animals were injected with JP.11 and JP.12, and the reactions were compared with a Compton heat-concentrated johnin (CJ.1) with the result shown in Table VII.

The reactions to JP.11 and JP.12 were very definite in the positive animals, whereas in the three which were negative there was no suspicion of a reaction on this occasion.

It is not possible to form any opinion from the results of this very

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restricted test on the value of P.P.D. johnin as a diagnostic agent for cattle, but, so far as it went, the evidence suggested that in a suitable strength P.P.D. is not less potent than a good heat-concentrated product.

TABLE VII

Comparison of Johnin P.P.D. (JP.11 *and* JP.12) *with Heat-concentrated Johnin* (CJ.1)

Cow.	*Johnin.*	*Initial measurement.*	*48th hour.*	*72nd hour.*	*Description of reaction.*	*Result.*
A	JP.11	7	12	16	D	R
	JP.12	7	10	16	D	R
	CJ.1	7	9	11	SD	?
B	JP.11	6	10	13	SD	R
	JP.12	6	8	13	SD	R
	CJ.1	5	6	9	bean	N
C	JP.11	6	14	18	D	R
	JP.12	6	13	18	D	R
	CJ.1	6	11	17	D	R
D	JP.11	7	13	18	D	R
	JP.12	7	13	18	D	R
	CJ.1	6	11	16	D	R
E	JP.11	6	7	8	N	N
	JP.12	6	6	7	N	N
	CJ.1	6	7	8	N	N
F	JP.11	6	6	6	N	N
	JP.12	6	6	7	N	N
	CJ.1	6	6	7	N	N
G	JP.11	7	9	8	N	N
	JP.12	7	7	7	N	N
	CJ.1	6	7	7	N	N

JP.11 = P.P.D. johnin (benzoic acid).
JP.12 = P.P.D. johnin (trichloracetic acid).
CJ.1 = heat-concentrated synthetic medium.
D = diffuse.
SD = slightly diffuse.
N = negative.

Discussion

Recent investigations on the purification of tuberculin culminating in the production of purified protein derivative have naturally aroused considerable interest in the possibility of obtaining products of a similar nature from other members of the acid-fast group. So far the methods devised by Seibert and modified by other workers have not been

extensively applied to Johne's bacillus. The reason for the slow progress is probably attributable to the difficulty in obtaining good growths of the organism on liquid medium without the aid of *M. phlei.* In this country Dunkin (1933) has shown that a suitable strain can be trained to grow freely on Henley's synthetic medium in the absence of phlei and will produce a johnin of good potency. In our experience it is vitally important to work with rapidly growing cultures of this type if satisfactory yields of johnin P.P.D. are to be obtained. With such cultures no great difficulty has been encountered in preparing protein derivatives of johnin comparable to those obtained from tuberculin, although the weight of dry powder has been smaller than that recorded by various workers for tuberculin protein. It is quite possible that the yields could be improved by a closer study of the optimal period of growth, constituents of the medium and other factors which influence the elaboration of the specific principle of johnin.

Even in this restricted study it has been found that some modifications of Seibert's method are desirable: for example, an appreciable amount of activity was lost when phenol water was used as a diluent prior to ultrafiltration. A similar loss of tuberculin protein has been reported by Jensen *et al.* (1938), who observed a reduction in activity of the final product of about one half. They have not offered any explanation of the phenomenon. It is likely, however, that the degree of loss is linked with the reaction of the johnin since a much higher proportion of precipitable substance developed at pH reactions of 6.2 to 5.2 than in solutions adjusted to 7.0 or over.

As a result of comparative trials in man, Long, Aronson and Seibert (1934) concluded that 1 mg. of O.T. was equivalent to 0.005 mg. of P.P.D.; on the other hand, Jensen *et al.* (1938) found that in the guinea-pig 1 mg. of O.T. caused as strong a reaction as 0.002 mg. of P.P.D. In this study a dose of between 3 and 4 mg. per ml. of P.P.D. has been equivalent to the heat-concentrated johnin selected as standard, which means that 1 mg. of standard johnin has a potency equivalent to 0.003 to 0.004 mg. of P.P.D. It is interesting that there is a fairly close parallelism between the comparison of tuberculin and johnin proteins with their homologous heat-concentrated products.

An encouraging feature of this work has been the relative uniformity in potency of successive batches of johnin irrespective of the method used for their preparation. It is true that the percentage amounts have shown appreciable variations but these may be attributable to fluctuations in the growth of the organism on liquid synthetic medium and to technical difficulties in the separation of the active principle. It is probable that these variations will be overcome and it is hoped that an active principle of johnin in a still higher state of purity will be obtained. At the same time it is not certain that further refinements in technique with a resulting improvement in purity would neces-

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sarily be followed by an increase in the potency of johnin or in its specificity as a diagnostic agent for the detection of Johne's disease in cattle. For example, Medlar *et al.* (1941), in common with other workers, have brought forward evidence showing that the protein fraction may not be the only substance in O.T. which produces a specific reaction, and cite certain data tending to show that O.T. is a slightly superior diagnostic agent to P.P.D. owing to the combined effect of tuberculin protein and other specific substances in the former. Jensen, *et al.* have also demonstrated that a comparison of a purified tuberculin and the international standard cannot be made by the standardizing method in the guinea-pig because the non-specific constituents of international tuberculin tend to increase the reaction. They found that when comparing P.P.D. with O.T. the calculated strength of the former differed from the value obtained by the biological test: they attributed this variation to the fact that non-specific substances in O.T. give rise to reactions which augment the specific reaction. This hypothesis was confirmed by the demonstration that P.P.D., to which concentrated glycerine veal broth had been added, induced reactions of a similar nature to those resulting from O.T.

In cattle, McIntosh (1939) states that, in an effort to remove the factors responsible for non-specific reactions, the technique described by Seibert was adopted for the preparation of johnin P.P.D. A purified product was obtained which gave well-marked reactions in sensitised guinea-pigs, while non-specific elements were practically eliminated. The results obtained by testing Johne-infected herds were said to be encouraging. Hopkirk (1939) obtained samples of johnin P.P.D. from Canada and has commenced some trials on infected herds in New Zealand. It is regretted that in this country an opportunity of testing P.P.D. johnin on significant numbers of known Johne-free and Johne-infected animals has not yet arisen. A few preliminary trials suggest that at a strength of 3 mg. per ml. johnin P.P.D. does not produce misleading reactions when applied by the double intradermal method, but no information is as yet available as to the best dose for use in the field. In view of the undoubted sensitivity to johnin of cattle affected with the avian bacillus, and also of the possible reactions of animals affected with the bovine type, it may be extremely important to select an amount of P.P.D. which, whilst it picks out the highest possible proportion of cattle suffering from Johne's disease, is unlikely to cause a reaction in those which are infected with bovine or avian strains.

Experiments on the rate of deterioration of johnin P.P.D. showed that, although there was a notable fall in potency of solutions stored at 37 deg. C., the stability of similar solutions at room temperature and in the cold room was of a relatively high order. From the practical point of view it is important to know the period over which solutions of johnin protein can be kept without an appreciable drop in active principle. It would seem that when stored under

proper conditions such solutions will not exhibit a significant loss in activity up to six months.

Conclusions

(1) Purified protein derivatives have been obtained from cultures of Johne's bacillus on liquid synthetic medium by ultrafiltration followed by precipitation with trichloracetic acid or adsorption on benzoic acid, and by saturation with ammonium sulphate. Each of these methods has provided dry, stable powders of good activity. The yield has ranged from 10.25 g. to 22.07 g. per 100 ml. of original fluid.

(2) The maximum amount of johnin P.P.D. which could be injected intradermally into normal guinea-pigs without producing a reaction was 0.001 g.

(3) The potency of johnin P.P.D. has been determined by the intradermal inoculation of guinea-pigs sensitized by the intraperitoneal inoculation of Johne's bacillus in liquid paraffin. In each case a comparison has been made with a sample of heat-concentrated synthetic medium johnin. In general it has been found that 0.1 g. of heat-concentrated johnin was equivalent to 0.0003 to 0.0004 g. of johnin P.P.D., irrespective of the method of its preparation. Sensitized guinea-pigs reacted to amounts of johnin P.P.D. ranging from 0.00012 mg. to 0.000074 mg.

(4) P.P.D. in solution containing not more than 3 mg. per ml. produced clear-cut negative reactions in cattle known to be free from Johne's disease. The same solution evoked œdematous swellings in reacting cattle which were as extensive as those resulting from a heat-concentrated synthetic medium johnin. The number of cattle tested, however, was very small.

(5) A solution of P.P.D. containing 5 mg. per ml. retained its activity unimpaired for a period of 175 days.

Acknowledgment

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DICEPHALUS IN A CALF

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THE subject was a brown heifer calf which had been born dead. A superficial examination of the calf suggested that the duplication was confined to the head region, the neck and the remainder of the body having a perfectly normal appearance. The two heads were equal in size and were joined together in the temporal region, their long axes diverging almost at right angles from one another. A white patch appeared on each forehead, the two patches being identical in outline. Four eyes were present. There were, however, only three ears, one lying laterally on each head in the usual position, and the third, which was very small and distorted, being situated between the two crania in the middle line. The two halves of the lower jaw of the left head were separated from one another at the symphysis by an interval, and there was a fissure in the free end of the corresponding tongue and lower lip (Fig. 1).

The only peculiarities in the neck region revealed in dissection concerned the large vessels. External and internal jugular veins were present on each side of the neck, and these drained the lateral aspects of the two heads, i.e., the left side of the left head and the right side of the right head. Below the trachea in the middle line ran another jugular vein equal in calibre to that of an external jugular vein. It drained the medial sides of the heads and it opened posteriorly into the right internal jugular vein a little in front of the jugular confluence (Fig. 2). Similarly there were three common carotid arteries arising from the brachiocephalic artery. Two lateral arteries ran up the neck on either side of the trachea as far as the pharynx, where they divided in the usual manner. The third artery accompanied the medial jugular vein, and, passing below the body of the hyoid bone, continued forwards to be distributed to the structures on the opposed surfaces of the two heads. The course of these vessels in the head will be described later.

On dissecting the heads (Fig. 3) considerable deviation from the normal was observed in the medial temporal regions as a result of their approximation. The medial masseter and pterygoid muscles were distorted and reduced in size. This was particularly the case on the left side, since the right mandible overlapped the left in this region. In front of this the facial muscles all presented the usual arrangement. Where the two intermaxillary spaces met one another in the middle line of the animal there was a fibrous raphe lying superficially and giving origin to the medial digastricus and mylo-hyoideus muscles of each head. The superficial layer of the mylo-hyoideus muscle of the left head appeared to be longitudinally divided.

FIG. 1.

MEDIAL ASPECT OF THE HEADS.

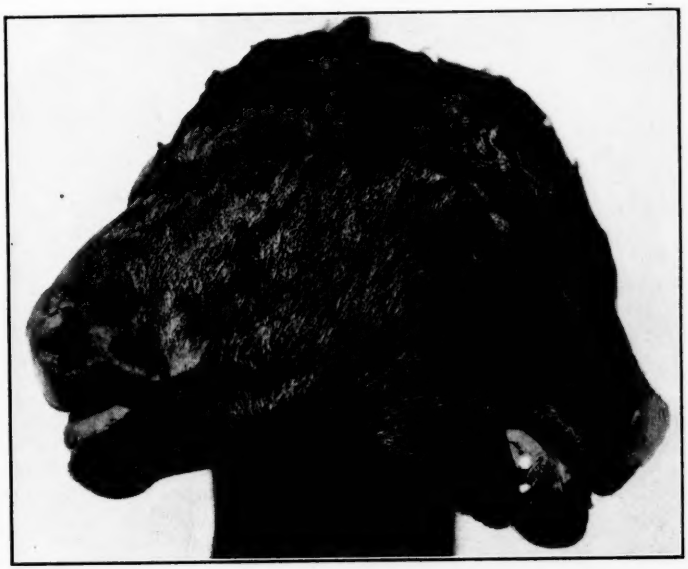
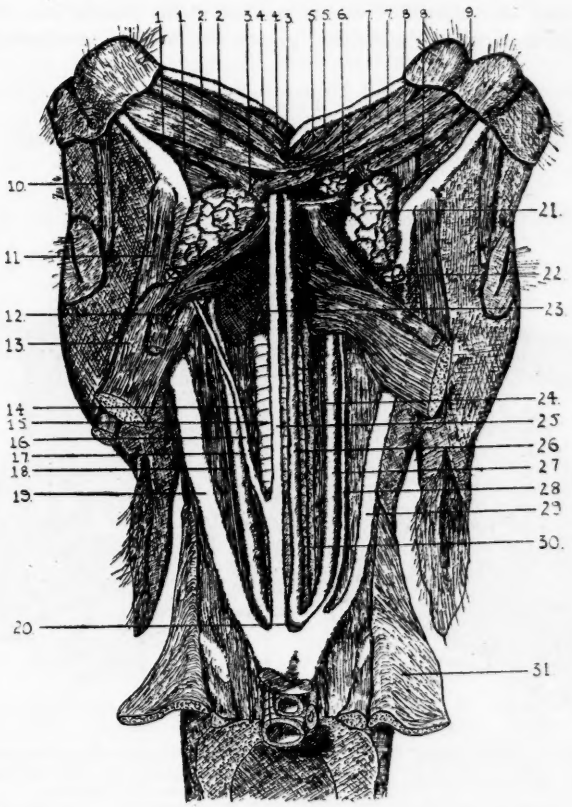


FIG. 2.

DISSECTION OF UNDER SURFACE OF NECK.



1. M. digastricus (lateral).
2. M. mylo-hyoideus.
3. Raphe between origins of M.m. mylo-hyoideus and digastricus (medial).
4. 5. M. digastricus (medial).
6. Left medial submaxillary salivary gland.
7. 8. Divisions of fissured M. mylo-hyoideus.
9. Area of incomplete symphysis mandibulæ.
10. M. zygomaticus.
11. M. sterno-mandibularis.
12. M. sterno-thyro-hyoideus.
13. M. sterno-mastoideus.
14. Recurrent laryngeal nerve.
15. Trachea.
16. Right lateral internal jugular vein.
17. Right lateral common carotid artery.
18. Right vago-sympathetic trunk.
19. Right lateral external jugular vein.
20. Brachiocephalic artery.
21. Left lateral submaxillary salivary gland.
22. Submaxillary lymphatic gland.
23. Thyroid gland.
24. Left vago-sympathetic trunk.
25. Medial jugular vein.
26. Medial common carotid artery.
27. Left lateral common carotid artery.
28. Left lateral internal jugular vein.
29. Left lateral external jugular vein.
30. Œsophagus.
31. M. scalenus.

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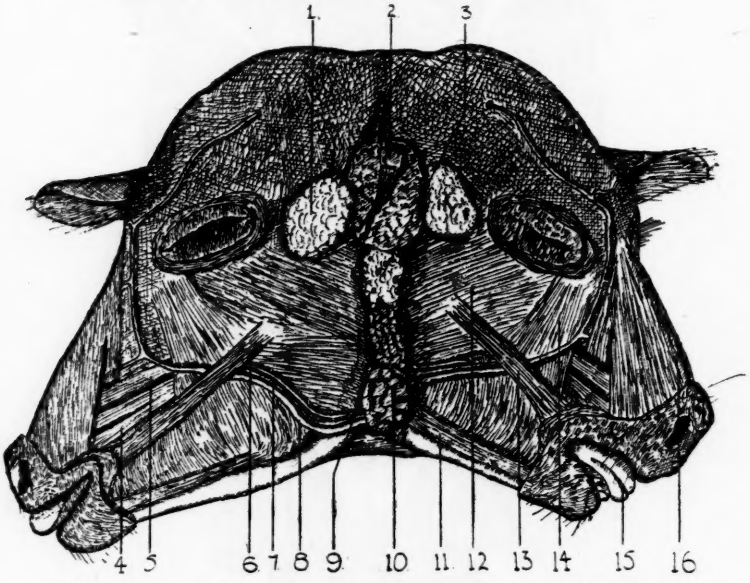
The medial parotid glands were small, and the left gland was situated above the right in the angle between the masseter muscles. From the glands the ducts ran a normal course. The medial submaxillary glands were also reduced in size and merged with one another in the middle line. These were the only abnormalities of the salivary glands which could be observed.

FIG. 3.

DISSECTION OF MEDIAL ASPECTS OF THE TWO HEADS.



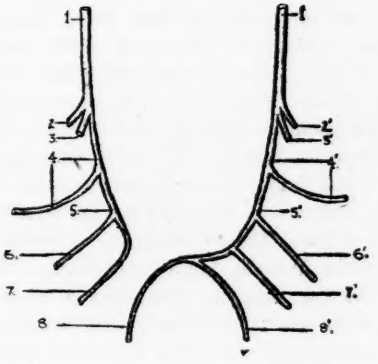
1. Mass of fat in temporal region.
2. Cartilage of common medial ear.
3. Mass of fat containing numerous lymphatic glands.
4. M. dilatator naris lateralis.
5. M. levator labii superioris proprius.
6. Parotid duct.
7. External maxillary artery.
8. External maxillary vein.
9. Parotid salivary gland of left head.
10. Parotid salivary gland of right head.
11. M. depressor labii inferioris.
12. M. masseter.
13. M. zygomaticus.
14. M. malaris.
15. Fissured tongue of left head.
16. M. levator labii superioris alæque nasi.

The arteries of the head showed several peculiarities (Fig. 4). On the right side the common carotid artery terminated by dividing into the usual three branches, viz., external carotid, occipital and external maxillary arteries. The last-named vessel was very large, this being due to the exceptional size of its lingual branch. This vessel after supplying the lateral aspect of the tongue continued under the root of this organ and divided into two branches, the right medial external maxillary artery and a vessel which continued across

the middle line of the animal as the terminal portion of the left medial external maxillary artery. On the left side the only peculiarity noted in connection with the common carotid artery was the fact that the external maxillary artery detached an additional lingual branch for the supply of the medial side of the left tongue. A third common carotid artery lay, as already mentioned, in the middle line of the neck. Arising from the brachiocephalic artery with the lateral carotid arteries this vessel passed up the neck with the medial jugular vein and on reaching the head ran forwards deep to the raphe, joining the

FIG. 4.

DIAGRAM OF ARRANGEMENT OF LATERAL COMMON CAROTID ARTERIES AND THEIR BRANCHES.



1., 1'. Common carotid arteries.
2., 2'. Occipital arteries.
3., 3'. External carotid arteries.
4., 4'. Lateral external maxillary arteries.
5., 5'. Large lingual branches of 4 and 4'.
6., 6'. Lateral lingual arteries.
7., 7'. Medial lingual arteries.
8., 8'. Medial external maxillary arteries.

mylo-hyoideus and medial digastricus muscles to gain the anterior aspect of the common pharynx. Here it divided into two occipital arteries and a single external carotid artery. The occipital arteries were represented by two vessels running to the anterior wall of the pharynx. The posterior meningeal branches arose as a single trunk detached from the external carotid close to the origin of the occipital arteries. The external carotid artery passed upwards deep to the medial parotid glands and just below the level of the temporo-maxillary joints divided into two internal maxillary arteries for the medial sides of the two heads, and a smaller trunk which soon divided into two superficial temporal arteries each running over the zygomatic arch of its own side into the temporal fossa.

On the lateral aspects of the neck there were internal and external jugular veins, the origin and course of which appeared to be quite normal. Another large jugular vein lay medially in the neck. It originated as a slender trunk in the region of the medial temporo-mandibular joints by the union of two superficial temporal veins. The vessel so formed ran deep to the medial parotid glands where it was joined by the two medial external maxillary veins. Thus it became quite a large vessel and it passed backwards, deep to the raphe joining the mylo-hyoideus and digastricus muscles, to the hyoid region, where it received branches from the submaxillary and parotid glands, and drained the greater part of both tongues. The vein then passed medially down the neck as already described. No medial internal jugular vein was present.

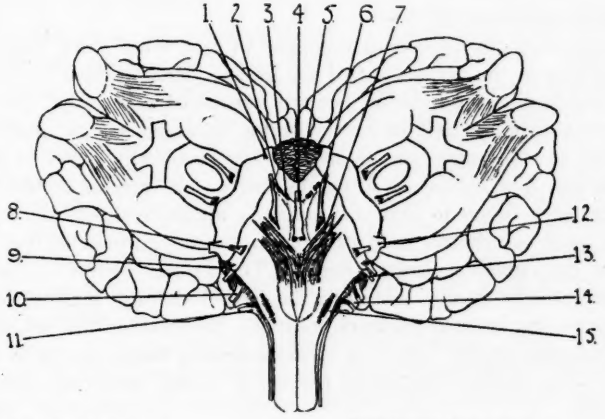
The anterior portions of the two mouths showed nothing peculiar apart from the fissure in the left tongue and lower lip already mentioned. Posteriorly the tongues merged and in this region there was a single foramen cæcum. No medial circumvallate papillæ were present on either tongue. The medial parts of the soft palates were ill-defined in conformity with the poor development of the tongues in this region. The two mouth cavities opened posteriorly into a common pharynx, the openings being separated by a sheet of muscle which was composed of what appeared to be the medial pterygo-pharyngei, the palato-pharyngei and a small band running transversely representing the stylo-pharyngei muscles. Below this sheet the medial stylo-glossi merged with one another in the middle line. Deeply seated to the stylo-glossi muscles and on the anterior wall of the pharynx lay a small elongated medial thyroid gland. The lateral walls of the pharynx presented a normal appearance. Four Eustachian tubes with associated tensor and levator palati muscles were present. A single hypoglossal nerve ran across the medial wall of the pharynx to supply the muscles of the medial surfaces of the two tongues.

The two skulls were united to one another along a vertical line passing through the posterior portions of the medial temporal regions. In this common part of the head there was a single occipital bone surrounding a normal foramen magnum. The basilar part of the bone bifurcated anteriorly to articulate on each side with a basi-sphenoid. In the angle of bifurcation, i.e., in the middle line, lay a single bone representing the combined petrous parts of the medial temporal bones. The squamous part of the single occipital bone, the four parietals and the two interparietals united to form an extensive curved plate which constituted the medial part of the roof of the common cranium. The plate lay between the four frontal bones laterally, running from the foramen magnum posteriorly to the medial temporal region anteriorly. It was not possible to detect with any certainty the presence of auditory processes on the fused petrous mass but on examining the interior of this bone a common tympanic cavity was found containing two tensor tympani muscles. The lower part of the bone exhibited a strong ridge representing the

laterally compressed auditory bullæ. The muscular processes were well developed. The squamous parts of the medial temporal bones were separate though much compressed. The remaining bones of each skull, both medial and lateral, presented no peculiarity with the exception of the mandibles which showed a considerable degree of distortion due to the approximation occurring in the temporo-maxillary regions and the over-lapping of the left mandible by the right here. The two halves of the left mandible were not united at the symphysis. There was a single hyoid bone which articulated above with the lateral petrous temporal bones in the usual manner.

FIG. 5.

VENTRAL ASPECT OF BRAINS.



1. Right medial trigeminal nerve.
2. Roots of right medial facial nerve.
3. Roots of right medial auditory nerve.
4. Single medial hypoglossal nerve.
5. Left cerebellum.
6. Left medial abducent nerve.
7. Right medial pyramid.
8. Right lateral abducent nerve.
9. Right lateral facial nerve.
10. Right lateral glosso-pharyngeal nerve.
11. Right lateral spinal accessory nerve.
12. Left lateral trigeminal nerve.
13. Left lateral auditory nerve.
14. Left lateral vagus nerve.
15. Roots of left lateral hypoglossal nerve.

Nothing abnormal was noted with regard to the foramina on the lateral sides of the skulls. The foramina of the medial sides, however, were considerably altered behind the level of the foramen ovale. There was a small foramen at the junction of the common mass of the petrous temporal bones and the basilar portion of the occipital bone in the middle line of the head which represented the combined condyloid foramina. On each side of this were small foramina representing the foramina lacera. Above each of these lay a small internal auditory meatus. The temporal canals opened inwardly on either side of the apex of the petrous mass, their external openings being

situated close together on either side of the ridge of bone representing the fused auditory bullæ.

The brain (Fig. 5), enclosed in a single sac of dura mater, showed a duplication of structures in front of the medulla. This latter became wide anteriorly and divided into two parts, each branch being margined in front by a pons which showed little alteration. On the base of the medulla, in the middle line, immediately anterior to the point of convergence of the pyramids a single large nerve originated. This was the hypoglossal nerve which gained exit from the cranium by the single condyloid foramen. Its further course has been described in connection with the pharynx and tongues. The medial corpora trapezoidea were evident on either side of this nerve with the superficial origins of the medial abducent nerves lying between the corpora and the pyramids. The origins of the facial and auditory nerves lay in front of the origins of the abducent nerves.

The two cerebella were closely applied to each other along their posteromedial surfaces in the middle line of the brain. Beneath them lay the fourth ventricle which, like the medulla, became duplicated anteriorly. In front of the pons the two brains and the cranial nerves appeared normal in all respects. None of the lateral cranial nerves showed any peculiarity either in its course or distribution. The medial facial nerves entered the internal auditory meatus where they were found to join each other at a common geniculate ganglion. From this latter two chorda tympani nerves arose which ran in the usual manner to join the corresponding lingual nerves. The further course of the seventh nerves could not be traced. Similarly the medial auditory nerve could not be traced beyond the internal auditory meatus. No trace of the origins of the medial glosso-pharyngeal, vagus, or spinal accessory nerves could be found on the brain. The medial hypoglossal nerves, as stated, were represented by a single nerve.

Although Dicephalus appears to be a not uncommon abnormality in the domesticated animals, a study of the literature on the subject reveals the fact that few specimens exhibiting the condition have been examined in detail. One reason for this may be that the specimen often arrives at the laboratory in a state of partial decomposition.

Dicephalus is, of course, a variety of Duplicitas anterior, and it is now generally agreed that the condition is the result of fission occurring in the very early stages of development of the embryo. A dissection is interesting in that it enables one to discover how far backwards the effect of the fission has extended and what are the relationships of structures in the parts of the body which have been duplicated to those which are single.

The problem is complicated by the fact that some of the effects of the primary factor of fission are obscured by those of a secondary factor, viz.,

growth in the middle line of the animal in the region of the point of bifurcation. Thus one finds that on either side of the line of fission structures may have developed as far as a certain stage when further growth is arrested owing to their being compressed the one against the other in the middle line. An instance of this is seen in the case of the medial facial nerves. These nerves ran a normal course within the cranium but in their passage through the common mass of the petrous temporal bones towards the middle line of the animal they merged and their development was arrested. Only their chorda tympani branches, which lay more laterally than the parent nerves, could be identified. Similarly the superficial origins of the medial 9th, 10th and 11th cranial nerves lying in the middle line of the animal at the point of division of the medulla had become suppressed, while the medial hypoglossal nerves, since they lay further from the central axis of the brain, were able to survive, although they were blended.

Whilst the fission occurred at a stage in the development of the embryo prior to the formation of the branchial arches, it is possible to form an opinion as to the extent of the fission in relation to them. It is obvious that the anlagen of the first branchial arches and clefts were involved in the fission for in each head there were completely developed jaws and Eustachian tubes. The extent of the area behind the first arch, however, is indefinite, but it would appear that both the second and third arches were affected in the fission though not altogether completely. Although the hyoid bone and larynx were single, the presence of facial muscles on the medial aspects of the two heads and of medial pharyngeal muscles suggest that the second and third arches were concerned in the duplication.

The presence of the medial common carotid artery can be explained by the fact that its branches originally arose from the aortic bulb, as branches of the medial branchial arch vessels, but due to retrogression of the heart the connection between the aortic bulb and these branches became elongated to form the artery.

In cases of cyclopia it has been suggested that the presence of some "inhibiting factor" in the surroundings of the embryo destroys the tissue in the anterior end of the embryo which normally separates the eyes. A similar "inhibiting factor" acting on an embryo at an earlier stage would conceivably cause a fission and since the cells would not be so specialised as to form one half or the other of the head, duplicitas would occur, partial or complete. In the latter case identical twins are the result. Cyclopia and duplicitas may thus have a common cause, the time at which the cause acts determining the condition which results.

Fission is regarded as being an attempt to increase the possibility of survival in response to adverse conditions. In the present case the left head also showed an attempt at fission, evidenced in the tongue and adjacent struc-

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tures. The adverse condition causing this may have been a slight preponderance of the right head over the left, which is indicated by the right mandible overlapping the left medially. This also may account for the two medial external maxillary arteries originating from the right common carotid.

The authors desire to express their gratitude to Professor J. F. Craig, of the Department of Veterinary Pathology, whose kindness in presenting to them the specimen made the dissection possible.

SUSPECTED SULPHANILAMIDE POISONING IN A CASE OF INCARCERATED INGUINAL PYOMETROCŒLE

By G. H. ARTHUR

University of Liverpool.

Subject

A TEN-YEAR-OLD crossbred terrier bitch, weighing 21½ lb.

History

The bitch had given birth to several litters of puppies, and the last œstrus had ended three weeks ago. Since then a large inguinal swelling had developed.

Symptoms

The general condition was fair and the temperature and pulse were not disturbed. The inguinal swelling was as large as a grapefruit, and was situated over the two left posterior mammary glands. Its contents were freely movable, but the enlargement could not be reduced by manipulation and pressure. There was no vulval discharge.

The diagnosis was one of irreducible inguinal hernia, and the radical operation was decided on.

After turning the bitch over to shave the operation site, a discharge was seen to be coming from the vulva. On pressing the swelling the discharge was increased. The diagnosis was now one of incarcerated inguinal pyometrocœle. Apparently the level of the vulva was too far above the level of the pus in the uterus for the latter to reach the exterior in the standing position, but by turning the animal on to its back the positions were reversed.

Operation

The bitch was anæsthetised by the intravenous injection of nembutal gr. 4.1. An incision four inches long was made just lateral to the last nipple down to the peritoneal sac. The latter was exposed by blunt dissection down to the inguinal ring. The sac was then opened and its contents seen to consist of the whole of the greatly distended uterus, two cystic ovaries and the broad ligaments. Ovaro-hysterectomy was then performed, but the right ovary could not be brought out sufficiently for ligation of the right ovarian vessels, and it was left *in situ*.

The ovarian ligatures were of No. 5 silk. The uterine stump was treated with tincture of metophen and then invaginated with a continuous Lembert suture of No. 00 catgut. After freeing the processus vaginalis from the hernial sac, the broad ligaments were returned to the abdomen, together with the right ovary and uterine stump. The inguinal sac was then twisted into a cord, ligated and divided near the inguinal ring. The subcutaneous tissues were approximated with a few sutures of No. 1 catgut and the skin with Halstead sutures of No. 1 silkworm gut.

During the operation 2 c.c. pituitrin were administered intramuscularly and 60 c.c. 10 per cent. glucose in normal saline injected subcutaneously.

On each of the next two days 60 c.c. glucose saline were injected subcutaneously, and milk was taken voluntarily. On the third day the temperature was 104.2 degrees, but the appetite was good. The operation wound was suppurating; several sutures were removed and the wound was irrigated.

The following day the temperature was 104 degrees. The bitch was fairly bright. The wound was irrigated, and at 4 p.m. gr. 37½ sulphanilamide (5 x 7½ gr. tablets) were given.

At 12 p.m. gr. 22½ sulphanilamide (3 x 7½ gr. tablets) were given.

The next morning the temperature was 101 degrees. The patient showed inco-ordination of movement, and after walking short distances, fell over on to her side with spasm of the extensor muscles of the limbs. Later she was unable to stand, became hyperæsthetic, and showed inco-ordinated paddling movements with the limbs rigidly extended. During the night the bitch succumbed.

Post-mortem Examination.—The most interesting feature was a well-marked chronic nephritis affecting both kidneys.

Discussion

Although the doses of sulphanilamide given were well within the limits of the recommended dosage, it is thought that the impaired renal function so interfered with the excretion of the drug as to cause a toxic concentration of sulphanilamide in the blood.

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Abstracts of Current Literature

Studies on Bovine Mastitis. I.—Studies of an Experimental Herd. From the Division of Animal Health and Nutrition, Council of Scientific and Industrial Research, Commonwealth of Australia. Bulletin No. 134. 1940. Melbourne.

MUCH has been written concerning the cause, course, prophylaxis and treatment of bovine mastitis. In this bulletin is contained a wealth of information on the subject, obtained from work carried out in an experimental herd specially built up from young heifers. A long summary contains most of the relevant findings; it is reproduced in full.

1. A milch herd was established by the selection of non-pregnant heifers with the addition of a few in the early stages of the first pregnancy. All animals were proved to be free from tuberculosis and contagious abortion before being admitted to the herd. The methods adopted in the establishment and general management of the herd are described in the paper.

2. Fifty heifers were secured, and thirty of those first coming into production were chosen as the experimental herd on which intensive bacteriological and clinical examinations have been made.

3. Bacteriological examinations of the milk from each quarter of the udder have been made at regular fortnightly periods, supplemented by special examinations in some instances. The results obtained during the first two lactation periods have been analysed and are summarized in the several sections.

4. During the first week in the lactation of each cow, bacteriological examinations were made daily.

(a) In the first lactation, 73 per cent. of the 119 functioning quarters were sterile during the first week. Appreciably high bacterial counts in the milk from the other quarters were present for one or more days and were due to non-hæmolytic micrococci which tended to disappear before the end of the week; in three of these quarters the infection persisted for the first seven days. *Str. agalactiæ* was recovered from sixteen quarters during the first week but persisted in no case.

(b) In the second lactation, 48 per cent. of the 118 functioning quarters were sterile during the first week. In the other quarters, infection persisted for various periods, being present for the first seven days in 46 quarters (39 per cent.). *Str. agalactiæ* was recovered from five quarters during the first week.

(c) There was no tendency for the bacterial status of the quarter in the

first week to be reflected in the bacterial status during the rest of the first lactation period. In the second lactation period, however, 72 per cent. of the quarters sterile in the first week remained sterile, and 95 per cent. of those with appreciable counts in the first week showed relatively high counts during the rest of the lactation period.

5. Bacteriological examinations were made at fortnightly intervals after the first week.

(a) During the first lactation period, 50 per cent. of the 119 quarters remained sterile. The remaining quarters gave appreciably high counts for various periods, eight of them throughout the whole period. For the most part the persistent high counts were due to non-hæmolytic micrococci, but hæmolytic staphylococci contributed to the high count in 17 quarters (14 per cent.) and *Str. agalactiæ* in nine quarters.

(b) During the second lactation period, 34 per cent. of the 118 quarters remained sterile. The remaining quarters gave appreciably high counts for various periods, 50 (42 per cent.) of them throughout the whole period. Non-hæmolytic micrococci were responsible for the majority of the persistent high counts, but hæmolytic staphylococci contributed to the high count in 22 quarters and *Str. agalactiæ* in two quarters.

(c) In each lactation period, infections by both micrococci and staphylococci tended to become permanent, but those due to *Str. agalactiæ* were transient.

6. (a) During the first lactation period *Str. agalactiæ* was recovered from the milk of 13 cows (43 per cent.) or 30 quarters (25 per cent.). It was recovered on a single occasion from 18 quarters, on two isolated occasions from six quarters, and in nine quarters the infection persisted for from two to 13 weeks. It produced clinical mastitis in two quarters.

(b) In the second lactation period, *Str. agalactiæ* was recovered from the milk of 21 cows (70 per cent.) or 43 quarters (36 per cent.). It was recovered on a single occasion from 28 quarters, on two isolated occasions from 11 quarters, on three or four isolated occasions from four quarters, and in two quarters the infection persisted for three and 14 weeks, respectively. It produced clinical mastitis in one quarter.

(c) These results are based on regular fortnightly examinations. When the examinations were made more frequently, the streptococcus was found more often and in a larger number of cows.

7. The strains of *Str. agalactiæ* (serological Group B of Lancefield) recovered from the herd have been classified by agglutination methods into two types, Type 1 and Type 7a. Type 1, which has predominated, has been divided into six subtypes. Type 7a has occurred slightly less frequently. Several strains have failed to fall into either of these types. On the basis of

the differentiation of the streptococci into types and subtypes, the data have been examined to determine if the chance of a cow's harbouring more than one type increases with an increase in the number of quarters involved or with an increase in the number of infections experienced. A statistically significant association has been found in each case.

8. Streptococci not belonging to the serological Group B have been isolated from time to time from the quarter samples of milk.

(a) During the first lactation period, these streptococci were recovered from 22 cows (73 per cent.) or 37 quarters (31 per cent.). They were recovered on a single occasion from 31 quarters, on two isolated occasions from five quarters, on three occasions from one quarter, and the infection persisted for four weeks in one quarter without producing clinical symptoms.

(b) In the second lactation period, they were recovered from 27 cows (90 per cent.) or 68 quarters (59 per cent.). They were recovered from 42 quarters on a single occasion, from 13 quarters on two isolated occasions, from eight on three occasions, and from five quarters on four, five, or seven occasions.

(c) Thus, these streptococci were recovered on 162 isolated occasions during the two lactation periods on the regular fortnightly examinations. In addition, 35 recoveries were made at supplementary examinations. Out of 163 of these isolations, Group II of Minett *et al.* has been recovered on 44 occasions.

(d) The incidence of these infections was similar to that of the Group B streptococci but, except in one case, these streptococci did not produce persistent infections, and at no time did they produce mastitis.

9. (a) During the first lactation period, hæmolytic staphylococci were recovered from the milk of nine cows (30 per cent.) or 17 quarters (14 per cent.). They produced recurrent infections in 10 quarters and more persistent infections in seven. Of the 17 quarters infected, six developed clinical staphylococcal mastitis.

(b) In the second lactation period, hæmolytic staphylococci were recovered from the milk of 14 cows (46 per cent.) or 22 quarters (18 per cent.). Recurrent infections occurred in 12 quarters, more persistent infections in eight, and, in two, staphylococci were found on a single occasion in each. Clinical mastitis was manifested in six quarters, in one of which the clinical symptoms continued from the first to the second lactation period.

10. Non-hæmolytic micrococci were found to be common invaders of the udder, but did not cause clinical mastitis.

(a) In the first lactation period, micrococci were recovered from the milk of 18 cows (60 per cent.) or 44 quarters (37 per cent.). Persistent infections

occurred in 32 quarters, recurrent infections in 71, whilst in four the occurrence was occasional and in one it was transient.

(b) In the second lactation period, micrococci were recovered from the milk of 26 cows (86 per cent.) or 76 quarters (64 per cent.). Persistent infections occurred in 61 quarters, recurrent infections in 13, and in two quarters micrococci occurred on a single occasion in each.

11. With one exception, the herd was free from udder infections by corynebacteria. Anærobic bacteria were not recovered from the milk of any of the cows.

12. *Str. agalactiæ* was recovered from the fæces of seven cows and from sores on the teats on two occasions.

13. Suckling calves appeared to have no appreciably beneficial or harmful effect as judged by the bacterial status of the udder. Overstocking during the drying-off period tended to increase the bacterial counts in the milk.

14. Attempts to control the bacterial status of the udder by sealing the teats were unsuccessful in the few cases treated.

15. Systematic bacteriological examinations at fortnightly intervals during the first lactation period of 12 first-calf heifers in another herd yielded results comparable with those obtained in the experimental herd.

16. The clinical investigations carried out on the experimental herd and the observations made on infective and other abnormalities of the udder are outlined. Sores on the udders and teats were common and appeared to be of staphylococcal origin. Case reports are given of three cows which developed streptococcal, and of seven cows which developed staphylococcal mastitis during one or other, or both, lactation periods. Bacterial infections in the quarters had no appreciable effect on the yield of milk unless clinical symptoms developed. Clots were found frequently in the milk from sterile quarters but were somewhat more frequent from quarters in which bacteria had become established.

Hypertrophic verrucose lesions of the lining membrane of the teat canal were frequent in occurrence. Of the 203 quarters under observation in both the experimental and non-experimental sections of the herd, 39 developed these lesions. They did not appear to be of bacterial origin.

The development of mastitis was not correlated with the productiveness of the cow nor with the size of the teat orifice.

17. The cellular content of the milk from quarters which remained free from bacterial infection remained low. Of the samples from these quarters, 92 per cent. yielded cell counts below 100,000 per ml., and 8 per cent. did not exceed this count.

Infected quarters yielded cell counts of 200,000 per ml., or more, in a large proportion of samples. The proportion of samples from quarters with

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pure micrococcal infections was 59 per cent., from those with pure staphylococcal infections it was 83 per cent., and from those with streptococcal infections it was 84 per cent.

18. The laboratory methods employed for the bacteriological, serological, and cytological examinations are outlined.

19. Attention is drawn to the failure of the methods adopted to enable the establishment of a herd free from infections by *Str. agalactiæ*. These preliminary results of the investigation are discussed. Attention is also drawn to the relatively high degree of infectivity of the micrococcus and the persistent character of the infections it has produced. The hæmolytic staphylococcus has exhibited an infectivity almost as high and the infections have also been very persistent. Infections by *Str. agalactiæ* have been more transient, and the results have failed to show that this streptococcus has a higher infectivity than the micrococcus or the staphylococcus.

The possible reasons for the failure of *Str. agalactiæ* to produce the typical chronic streptococcal mastitis described by other workers are discussed. It is concluded that the possible absence of "epidemic strains" of streptococcus, the absence of preliminary damage to the tissues of the udder by other infections on account of the youth of the cows, or both, may have been responsible for the more transient nature of the infections.

The results have shown that the infected udder is not the only reservoir of *Str. agalactiæ*, and they suggest that methods of control of streptococcal mastitis based on the assumption that it is the only reservoir may meet with only partial success.

Spread of Infection from the Respiratory Tract of the Ferret. I.—Transmission of Influenza A Virus. C. H. ANDREWES and R. E. GLOVER. *Brit. J. Exp. Path.* 1941. *XXII*, 91. II.—Association of Influenza A Virus and Streptococcus Group C. R. E. GLOVER. *Brit. J. Exp. Path.* 1941. *XXII*, 98.

I.—IN this article are recorded the results of experiments which indicate that the infection-bearing particles in influenza A of ferrets are not exclusively the gross droplets which are expelled when the animal sneezes. It was shown that infection occurred over a distance of over five feet in almost still air. It is not denied that coarse droplets may convey infection but the work strongly suggests that very fine particles may also convey infection. Increased ventilation or the interposition of a screen between the ferrets appeared to decrease the chances of cross-infection.

II.—This article deals with ferrets doubly infected with influenza A virus and streptococci. A method of inducing in the ferret a nasal infection by the combined action of influenza A virus and a hæmolytic streptococcus was

devised—it was found that the infection could be brought about by administering the streptococcus up to seven days after the virus. It was also shown that the streptococcus alone without the presence of the virus was incapable of infecting the nasal passages.

These findings are of particular importance in that they open the way for a more detailed study of air-borne transmission of infection and we are promised more information on the subject in future communications.

News

AGRICULTURAL RESEARCH COUNCIL

Sir Edwin Butler, F.R.S., the Secretary of the Agricultural Research Council since 1935, has resigned, through ill health, and has been succeeded by Professor W. W. C. Topley, M.D., F.R.S.

Professor Topley was Professor of Bacteriology and Immunology in the University of London and Director of the Division of Bacteriology and Immunology of the London School of Hygiene and Tropical Medicine. He is well known to veterinary research workers for his interest in problems relating to diseases of animals and for the standard text-book, "The Principles of Bacteriology and Immunity," prepared by him and Professor G. S. Wilson.

AGRICULTURAL IMPROVEMENT COUNCIL

The following abstract is taken from *The Times* of June 13, 1941 :—

HOUSE OF COMMONS

Thursday, June 12

The Speaker took the Chair.

Agricultural Research.—Replying to Earl Winterton, Mr. R. S. Hudson, Minister of Agriculture (Southport, U.), said that he had appointed a body which would be known as the "Agricultural Improvement Council for England and Wales" with the following terms of reference: "To devise methods for seeing that promising results of research are applied as rapidly as possible to the problems of agriculture and are incorporated in ordinary farming practice; and to advise from time to time concerning agricultural problems which appear to require scientific investigation."

The Council would consist of a chairman and 19 members, and the term of appointment would be for three years with the possibility of reappointment. The following were the name of the Committee: Sir Donald Fergusson, K.C.B., Fermanent Secretary of the Ministry of Agriculture and Fisheries (chairman); Mr. Dennis Brown, farmer; Lord Cornwallis, M.C., Chairman of Kent War Agricultural Executive Committee; Professor T. Dalling, M.A., M.R.C.V.S., Institute of Animal Pathology, Cambridge; Professor F. L. Engledow, C.M.G., School of Agriculture, Cambridge; Captain E. Foster, O.B.E., Chairman of Shropshire War Agricultural Committee; Mr. A. Holness, Editor of the *Journal* of the National Union of Agricultural Workers; Mr. C. Nevile,

farmer, Vice-President of N.F.U.; Professor J. A. Scott Watson, School of Rural Economy, Oxford; Professor Sir R. George Stapledon, C.B.E., M.A., F.R.S., Welsh Plant Breeding Station, Aberystwyth; Professor W W. C. Topley, F.R.S., Secretary of Agricultural Research Council; Mr. L. G. Troup, Agricultural Organizer and Executive Officer of Hants War Agricultural Executive Committee.

Mr. Hudson added that he had invited the Earl of Feversham to be a member of the Council on his return from active service abroad. The technical secretaries of the Committee were: Mr. J. C. F. Fryer, C.B.E., and Mr. E. C Lloyd, M.R.C.V.S.; and the administrative secretary Mr. W. R. Black.

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